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Short communication

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SREBP1, PPARG and AMPK pathways mediated the Cu-induced change in intestinal lipogenesis and lipid transport of yellow catfish *Pelteobagrus fulvidraco*

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Abstract

Cu could act as a modifier and influence lipid metabolism, but the potential mechanism was not explored. Juvenile yellow catfish were fed diet containing 0.71 (low Cu), 3.93 (intermediate Cu) and 88.81 (high Cu) mg Cu kg⁻¹, for 8 weeks to explore the modulation of intestinal lipid metabolism following dietary Cu addition. Using specific pathway inhibitors (Fatostatin for SREBP1, T0070907 for PPARG and Compound C for AMPK), primary enterocytes of yellow catfish were used to explore the molecular mechanisms of Cu reducing intestinal lipid deposition. Dietary Cu addition triggered Cu accumulation but suppressed lipid deposition in the fore- and mid-intestine. The reduced lipid deposition was attributable to the suppressed lipogenesis and lipid absorption, and accelerated lipid transport. The PPARG, SREBP1 and AMPK signaling pathways mediated the Cu-induced changes in lipogenesis, lipid uptake and lipid transport in the intestine of yellow catfish.

Keywords: Copper; Intestine regionalization; Lipid metabolism; Signaling pathways;

Vertebrate animals

Abbreviations

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ANOVA, one-way analysis of variance; Apo, apolipoprotein; CC, Compound C; CD36, fatty acid translocase; CM, chylomicrons; Cu, copper; F, Fatostatin; FABP, fatty acid-binding protein; FAS, fatty acid synthase; FATP, Fatty acid transport protein; FBW, final mean body weight; FI, feed intake; G6PD, glucose 6-phosphate dehydrogenase; H&E, hematoxylin-eosin; ICDH, isocitrate dehydrogenase; ICP-AES, inductively coupled plasma atomic emission spectrometry; IBW, initial mean body weight; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; ME, malic enzyme; PPAR, peroxisome proliferators-activated receptor; qPCR, real-time fluorescence quantitative PCR; SEM, standard error of means; SGR, specific growth rate; SR, survival rate; SREBP, sterol-regulator element-binding protein; T, T0070907; TG, triacylglycerol; WG, weight gain.

1. Introduction

Copper (Cu) is a vital trace element for all animals, including fish. It is co-factor of many enzymes and plays important roles in many physiological processes of animals (Watanabe et al., 1997). Studies have shown that dietary Cu deficiency reduced appetite and growth performance (Tan et al., 2011; Chen et al., 2015; Wang et al., 2016). In contrast, excessive Cu in diet can be toxic and cause growth retardation, oxidative stress and intestine damage (Chen et al., 2015; Wang et al., 2016).

Lipids are critical to maintain cellular energy homeostasis and serve a vast array of functions in animals, including fish. Lipid deposition and metabolism are complex processes, involved in the balance among lipogenesis, lipolysis, lipid absorption and transport. In mammals, studies showed that Cu acted as a modifier and influence lipid metabolism (Burkhead et al., 2013). In contrast, in fish, mechanistic studies on how Cu altered lipid metabolism was very scarce. Recently, Chen et al. (2015) investigated the Cu-induced changes in activities and mRNA expression of enzymes and genes involved in lipid metabolism (such as G6PD, 6PGD, ACC, FAS, PPAR α , PPAR γ and SREBP-1) in three tissues of yellow catfish, but not in the intestine. However, Chen et al. (2015) did not investigate the Cu-induced changes of lipid uptake and transport, and of the related signaling pathways. In fish, the intestine is the main organ for dietary Cu absorption (Handy, 1992), and lipid absorbed by the intestine is mainly derived from the diet. However, in fish, limited information was available on effects of dietary Cu levels on the intestinal lipid metabolism. Moreover, different intestinal regions possessed different characteristics of lipid absorption and transport because of their marked anatomical differences. Thus, it will be very interesting and challenging to investigate these differences in intestinal regionalization of fish fed different mineral (also Cu) diets. To this end, the present study was conducted to determine the effect of dietary Cu levels on fore- and mid-intestinal lipid deposition and metabolism in yellow catfish *Pelteobagrus fulvidraco*, which are omnivorous freshwater fish and widely cultured in China and other Asian countries. Primary enterocytes of yellow catfish were used to explore the potential signaling pathways for the Cu-induced changes of intestinal metabolic responses.

2. Materials and methods

The experiment performed on animals based on the Guide of Huazhong Agricultural University for the care and use of laboratory animals. In Expt. 1 and Expt. 2, Cu, in the form of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, was added.

2.1. Expt. 1: in vivo study

2.1.1. Diet preparation

Three experimental diets were formulated with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ supplemented at levels of 0, 0.013 and 0.39 g kg^{-1} diet at the expense of cellulose (Tan et al., 2011; Cheng et al., 2017), as shown in Supplementary Table 1. Dietary Cu concentrations were analyzed in triplicate using ICP-AES, and the contents were 0.71 (low Cu), 3.93 (intermediate Cu), and 88.81 (high Cu) mg Cu kg^{-1} diet.

2.1.2. Experimental procedure

The experimental procedures were similar to our previous study (Chen et al., 2015) as described in Cheng et al. (2017). Briefly, 216 juvenile yellow catfish (initial mean weight: 0.78 ± 0.01 g, mean \pm SEM, mixed sex) were randomly divided into 9 tanks, 24 fish per tank. They were provided with three experimental diets, three replicate tanks for each diet, for 8 weeks. Fish were fed to apparent satiation twice daily during the experiment. The water in the tanks was continuously aerated to maintain the dissolved oxygen level near saturation. During the experiment, water was renewed twice daily to maintain good quality.

After the 8-wk feeding experiment, 24 h after the starvation, fish were euthanized (MS-222 at 100 mg/l), counted and weighed to determine survival, WG and SGR. Then, fish were dissected, and the contents of the intestine were gently scraped off on ice. The fore-intestine (from the end of the stomach to the first loop of the intestine) and mid-intestine (from the first loop to the last loop) were used for the

following analysis, including Cu and TG contents, enzymatic activities and mRNA expression.

2.2. Expt. 2: *in vitro* study

2.2.1. Cell isolation and cultures

Enterocytes were isolated from yellow catfish based on the methods of Chen et al. (2009). Using primary enterocytes of yellow catfish, 3 independent experiments were conducted. In Expt. 2A, primary enterocytes received 1 of 4 treatments: control (0.01% DMSO), 10 μ M Cu, 10 μ M Fatostatin (specific inhibitor for SREBP1), 10 μ M Fatostatin, 10 μ M Cu+10 μ M Fatostatin. In Expt. 2B, primary enterocytes received 1 of 4 treatments: control (0.01% DMSO), 10 μ M Cu, 1 μ M T0070907 (specific inhibitor for PPARG), 10 μ M Cu +1 μ M T0070907. In Expt. 2C, primary enterocytes received 1 of 4 treatments: control (0.01% DMSO), 10 μ M Cu, 1 μ M Compound C (specific inhibitor for AMPK), and 10 μ M Cu +1 μ M Compound C. The inhibitors were provided 2 h before Cu incubation. Based on the published literatures and our pilot studies, we determined the suitable concentrations of Cu and specific inhibitors (Lee et al., 2002; Wu et al., 2016; Chen et al., 2017).

2.3. Sample analysis

2.3.1. Cell viability and contents of Cu, TG and ATP

Cell viability and contents of Cu, TG and ATP were quantified based our published methods (Liu et al., 2010; Wu et al., 2016). Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. TG content was measured by glycerol-3-phosphate oxidase p-aminophenol (GPO-PAP) methods using a commercial kit (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China). ATP content was analyzed using an ATP Assay Kit

(Beyotime, Haimen, China). Cu content was examined by the method of ICP-AES.

2.3.2. Enzymatic activities analysis

Five intestinal lipogenic enzymes activities, such as 6PGD, G6PD, ME, ICDH and FAS, were determined as described in the study by Chen et al. (2015). One unit of enzyme activity was defined as 1 μ M of substrate converted to product per minute at 28 °C and was expressed as mU mg⁻¹ soluble protein. The protein concentration was determined using BSA as standard (Bradford, 1976).

2.3.3. Gene expression analysis

Gene expression level was examined using real-time quantitative fluorescence PCR (qPCR) (BIO-RAD, USA) as described by Chen et al. (2015). A specific pair of PCR primers for every gene were given in Supplementary Table 2. The qPCR conditions were followed: 95°C for 30s, followed by 40 cycles at 95°C for 5s, 57°C for 30s and 72°C for 30s. By the detection of *β -actin*, *gapdh*, *rpl7*, *b2m*, *hpert*, *elfa* and *tuba*, we selected the most stable two genes as endogenous control by the geNorm software under the experimental conditions. The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

2.4. Statistical analysis

Statistical analyses were analyzed by the SPSS 19.0 software and presented as mean \pm SEM. Kolmogorov-Smirnov test was used for testing the normal distribution of all data. Bartlett's test was performed for testing the homogeneity of variances. One-way ANOVA and Duncan's multiple range test were used to analyze the differences among three groups. The interaction of Cu and inhibitors in the *in vitro* studies were performed by two-way ANOVA. It was considered significant when $P < 0.05$.

3. Results and Discussion

3.1. Expt. 1: *in vivo* study

3.1.1. Growth performance and Cu contents

WG, SGR and FI were the highest but FCR was the lowest in intermediate Cu group and did not show marked changes between other two groups (Table 1), indicating that yellow catfish fed low- and high Cu diets showed growth restriction and lower feed utilization, in agreement with our earlier reports (Tan et al., 2011; Chen et al., 2015). Survival was the lowest for fish fed high dietary Cu and showed no marked changes between other two groups, revealing that excessive Cu was toxic for yellow catfish. In the fore- and mid-intestine, Cu contents increased with increasing dietary Cu levels (Fig. 1A), similar to other reports (Lundebye et al., 1999; Shaw et al., 2006).

3.1.2. Intestinal TG contents, lipogenic enzyme activities and genes expression

Intestinal TG contents declined with increasing dietary Cu levels (Fig. 1B). In the fore-intestine, dietary Cu addition tended to reduce activities of ICDH, FAS, G6PD and ME (Fig. 1C), down-regulated mRNA levels of *6pgd*, *g6pd*, *fas*, *fatp4*, *fabp2* and *cd36*, and up-regulated mRNA levels of *mtp*, *apoi*, *apob* and *lpl*. *acca* mRNA expression was unaffected by dietary Cu addition (Fig. 2). In the mid-intestine, dietary Cu addition tended to reduce activities of ICDH and FAS (Fig. 1C), and down-regulated mRNA levels of *6pgd*, *g6pd*, *fas*, *acca*, *fatp4*, *fabp2* and *cd36*, and up-regulated mRNA levels of *mtp*, *apoi*, *apob* and *lpl* (Fig. 2). In contrast, G6PD and ME activities in the mid-intestine were lowest for intermediate-Cu diet group. G6PD, 6PGD, ICDH, ME, FAS and ACC are the important lipogenic enzyme and directly

involved in fatty acids biosynthesis (Elliot and Elliot, 2009). Dietary Cu-induced reduction in activities of lipogenic enzymes and gene expression would contribute to the reduction in lipogenesis, consistent with reduced TG content. *fatp4*, *fabp2* and *cd36* are important factors in the FA transporters (Lagakos et al., 2011; Sánchez-Gurmaches et al., 2012). Cu-induced reduction in mRNA expression of *fatp4*, *fabp2* and *cd36* was assumed to reduce fatty acids uptake, which would in turn reduce TG content. In fish, similar to mammals, lipids are exported from the intestine in the form of lipoproteins (Kjær et al., 2009). ApoAI, ApoB, MTP and LPL participated in lipoprotein metabolism and played important roles in intestinal lipid transport (Nilsson-Ehle et al., 1980; Hussain et al., 2003; Gu et al., 2014). In the present study, dietary Cu-induced up-regulation of mRNA levels of *apoai*, *apob*, *mtp* and *lpl* in the fore- and mid-intestine indicate that the decrease in intestinal TG content might partially be due to the fact that Cu accelerated lipid transport in the intestine.

In the fore-intestine, compared to fish fed low-Cu diet, dietary Cu addition down-regulated mRNA levels of *pparg* and *srebp1*, and up-regulated mRNA levels of *ampka1*, *ampkb1*, *ampkb2*, *ampkg1* and *ampkg2*. mRNA expressions of *ppara* and *ampka2* were unaffected by dietary Cu addition (Fig. 2). In the mid-intestine, compared to fish fed low-Cu diet, dietary Cu addition down-regulated mRNA expression of *pparg* and *srebp1*, up-regulated mRNA levels of *ampka1*, *ampka2*, *ampkb1* and *ampkg1*. mRNA levels of *ppara*, *ampkb2* and *ampkg2* were not significantly affected dietary Cu addition (Fig. 2). PPAR α , PPAR γ and SREBP 1 are key transcriptional factors which regulate lipid metabolism (Zheng et al., 2013; Wei et al., 2017). AMPK acts as an energy sensor and regulator of energy balance at the cellular and whole-body levels (Wu et al., 2017). Thus, Cu-induced changes of mRNA expression of *pparg*, *srebp1* and *ampk* family members indicated that they

potentially mediated the Cu-induced regulation of lipid metabolism.

3.2. Expt. 2: *in vitro* study

3.2.1. Cell viability, TG and ATP contents

In order to further confirm whether *pparg*, *srebp1* and *ampk* mediated the Cu-induced regulation of lipid metabolism, Fatostatin (specific inhibitor for SREBP1), T0070907 (specific inhibitor for PPARG) and CC (specific inhibitor for AMPK) were used and primary enterocytes of yellow catfish were cultured. Our study indicated that cell viability was unaffected by these inhibitors (Fig. 3A). Co-treatment with Cu and T0070907 or Compound C interacted (P -interaction < 0.05) to affect TG and ATP contents (Fig. 3B). Cu incubation reduced intracellular TG and ATP contents (Fig. 3B), and enterocyte TG content was unaffected by single Fatostatin, T0070907 or Compound C. However, Fatostatin or T0070907 pre-treatment accentuated the Cu-induced reduction in TG content, and CC pre-treatment alleviated the Cu-induced reduction in TG content. CC pre-treatment also significantly reduced the intracellular ATP content, but CC pre-treatment alleviated the Cu-evoked reduction in ATP content (Fig. 3C). These results supported that *pparg*, *srebp1* and *ampk* family members mediated the Cu-induced regulation of lipid metabolism.

3.2.2. Enzymatic activities and genes expression

In order to determine the mechanism of *pparg*, *srebp1* and *ampk* pathways mediated the Cu-induced regulation of lipid metabolism, we further determined many key enzymes and genes involved in lipid metabolism. Cu and Fatostatin co-treatment interacted (P -interaction < 0.05) to affect activities of ME and FAS (Fig. 4A), and the mRNA expression of *acca*, *fas*, *fabp2*, *mtp* and *srebp1* (Fig. 5A). Compared to the Cu-incubated group, Fatostatin pre-treatment accentuated Cu-induced reduction of

activities of ICDH, ME and FAS, accentuated Cu-induced reduction of mRNA concentrations of *acca*, *fas*, *fabp2* and *srebp1*, and accentuated Cu-induced up-regulation of *mtp* and *lpl* mRNA concentrations, which would help reduce lipid content in enterocytes. SREBP-1 is involved in FA synthesis (Horton and Shimomura, 1999). The Cu-induced down-regulation of *srebp1* expression, as observed in the present study, would reduce lipid deposition in the intestine by decreasing lipid synthesis, as reported by Horton and Shimomura (1999). Similarly, Chen et al. (2017) found that Fatostatin pre-treatment down-regulated mRNA concentrations of *6pgd*, *me*, *fas*, *acc* and *srebp1* in the hepatocytes of *Synechogobius hasta* (marine teleost) exposed to Fe. Thus, these observations indicated that Cu-evoked alterations in lipid metabolism, at least in part, was via the SREBP1 pathways.

The present study indicated that co-treatment with Cu and T0070907 interacted (P -interaction <0.05) to affect activities of G6PG, ICDH and FAS (Fig. 4B) and mRNA concentrations of *acca*, *fas*, *fatp2*, *cd36*, *apob*, *lpl* and *pparg* (Fig. 5B). Compared to the Cu-incubated group, T0070907 pre-treatment accentuated Cu-induced reduction of ICDH, ME and FAS activities, accentuated the Cu-induced reduction of mRNA concentrations of *fas*, *fabp2*, *cd36*, and *pparg*, and accentuated the Cu-induced up-regulation of mRNA concentrations of *mtp*, *apob* and *lpl*. Considering that PPARG regulates the expression of the genes coding for lipogenic enzymes and mediates TG synthesis (Chen et al., 2015), the present study indicated that PPARG pathway mediated the observed changes in lipid deposition induced by Cu. Most importantly, our data provided direct evidence that PPARG pathways also mediated intestinal lipid uptake and transport.

In the present study, co-treatment with Cu and Compound C interacted (P -interaction <0.05) to affect activities of ICDH, ME and FAS (Fig. 4C), and the

mRNA concentrations of *6pgd*, *g6pd*, *acca*, *fas*, *fabp2*, *cd36*, *mtp*, *apoai*, *apob*, *ampkb1* and *ampkg2* (Fig. 5C). Compared with the Cu-incubated group, Compound C pre-treatment significantly alleviated Cu-induced down-regulation of the lipogenic enzymes' activities (ICDH, ME and FAS) (Fig. 4C) and genes expression (*6pgd*, *g6pd*, *acca*, *fas*) as well as lipid absorption-related genes expression (*fatp4*, *fabp2*, *cd36*), and reduced the Cu-induced increase of lipid transport-related gene expression (*mtp*, *apoai*, *lpl*) and mRNA levels of AMPK subunits (*ampkb1*, *ampkg2*) (Fig. 5C). Thus, the Cu-induced up-regulation of gene expression of AMPK members may constitute a link between Cu and enzymatic activities/ genes expression in yellow catfish. The AMPK pathway acts as an intracellular regulator in energy homeostasis, and is activated by ATP depletion or increasing AMP/ATP ratio (Wu et al., 2016). The activation of AMPK pathway was likely attributable to cellular ATP depletion, as shown in the reduction of ATP content, also in agreement with the report by Wu et al. (2016). Other studies indicated that the changes in lipid metabolism after AMPK activation were accompanied by the reduced ACC activity in rats and mice, and decreased TG content in hepatocytes of rats (Muoio et al., 1999; You et al., 2004). Taken together, AMPK pathway took part in the activation of Cu-induced variation in lipogenesis, lipid absorption and lipid transport.

In conclusion, our study demonstrated that dietary Cu addition triggered Cu accumulation but lower TG storage in the fore- and mid-intestine of yellow catfish. The reduced lipid deposition was attributable to the suppressed lipogenesis and lipid absorption, and accelerated lipid transport. PPARG, SREBP1 and AMPK pathways mediated the Cu-induced changes in lipogenesis, lipid absorption and lipid transport in enterocytes of yellow catfish.

Conflict of Interest

The authors declare no conflicts of interest with the contents of this article.

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Contributions of authors are as follows: Z. L and G-H. C. designed the experiment and analyzed the data with the help of Hogstrand, C.; K. W. conducted the vitro experimental; S-C. L and. D-G. Z.: assisted with conducting the feeding trial and sample analysis; Y-H. X. helped with analysis of gene expression and enzymatic activities; Z. L. and Hogstrand, C. revised the manuscript; and all authors read and approved the final manuscript.

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Table 1Effect of dietary Cu levels on growth performance of yellow catfish after 8 weeks¹.

	Low Cu (0.71 mg Cu/kg)	Intermediate Cu (3.93 mg Cu/kg)	High Cu (88.81 mg/Cu kg)
IBW, g/fish	0.79 ± 0.03	0.77 ± 0.01	0.77 ± 0.02
FBW, g/fish	1.79 ± 0.03	1.87 ± 0.01 ^b	1.71 ± 0.05
WG ² , %	125.40 ± 6.40 ^a	144.22 ± 5.59 ^b	122.30 ± 2.60 ^a
SGR ³ , % day ⁻¹	1.80 ± 0.06 ^a	1.98 ± 0.05 ^b	1.78 ± 0.03 ^a
FI, g/fish	1.96 ± 0.05 ^{ab}	2.01 ± 0.01 ^b	1.89 ± 0.02 ^a
FCR ⁴ , %	1.96 ± 0.02 ^{ab}	1.83 ± 0.04 ^a	2.01 ± 0.07 ^b
SR ⁵ , %	90.00 ± 1.90 ^b	91.11 ± 1.11 ^b	84.40 ± 1.10 ^a

¹Values are mean ± SEM (n = three replicate tanks). Different letters indicate significant differences among three treatments ($P < 0.05$).

IBW, initial mean body weight; FBW, final mean body weight; FI, feed intake; SR, survival rate; WG, weight gain.

²WG = $100 \times (\text{FBW} - \text{IBW}) / \text{FBW}$;

³SGR = $100 \times (\ln(\text{FBW}) - \ln(\text{IBW})) / \text{days}$;

⁴FCR = $\text{FI} / (\text{FBW} - \text{IBW}) + (\text{dead fish weight} - \text{IBW})$;

⁵SR = $100 \times (\text{final fish number}) / (\text{initial fish number})$.

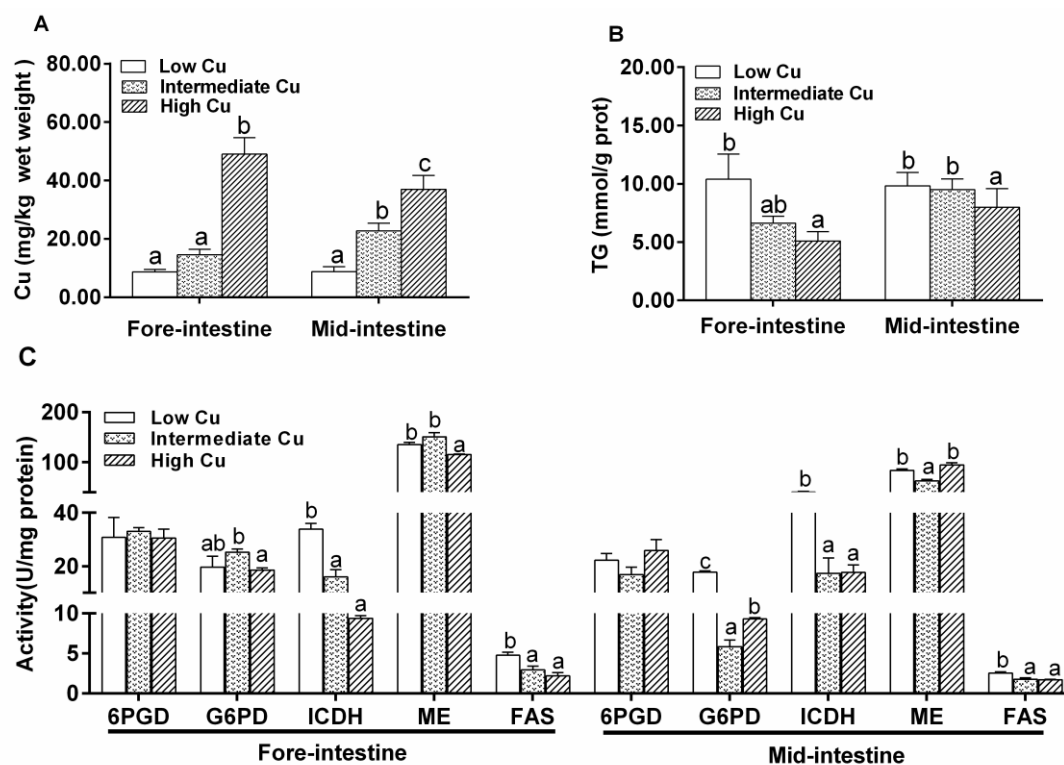


Fig. 1. Effect of different dietary Cu levels on Cu content (A), TG content (B) and enzyme activities (C) in the in the fore- and mid-intestine of yellow catfish. Values are mean \pm SEM ((n= three replicate tanks. For Cu content analysis, 4-6 fish sampled for each tank; For TG content and enzyme activity, six fish were sampled for each tank). Different letters indicate significant differences among three treatments.

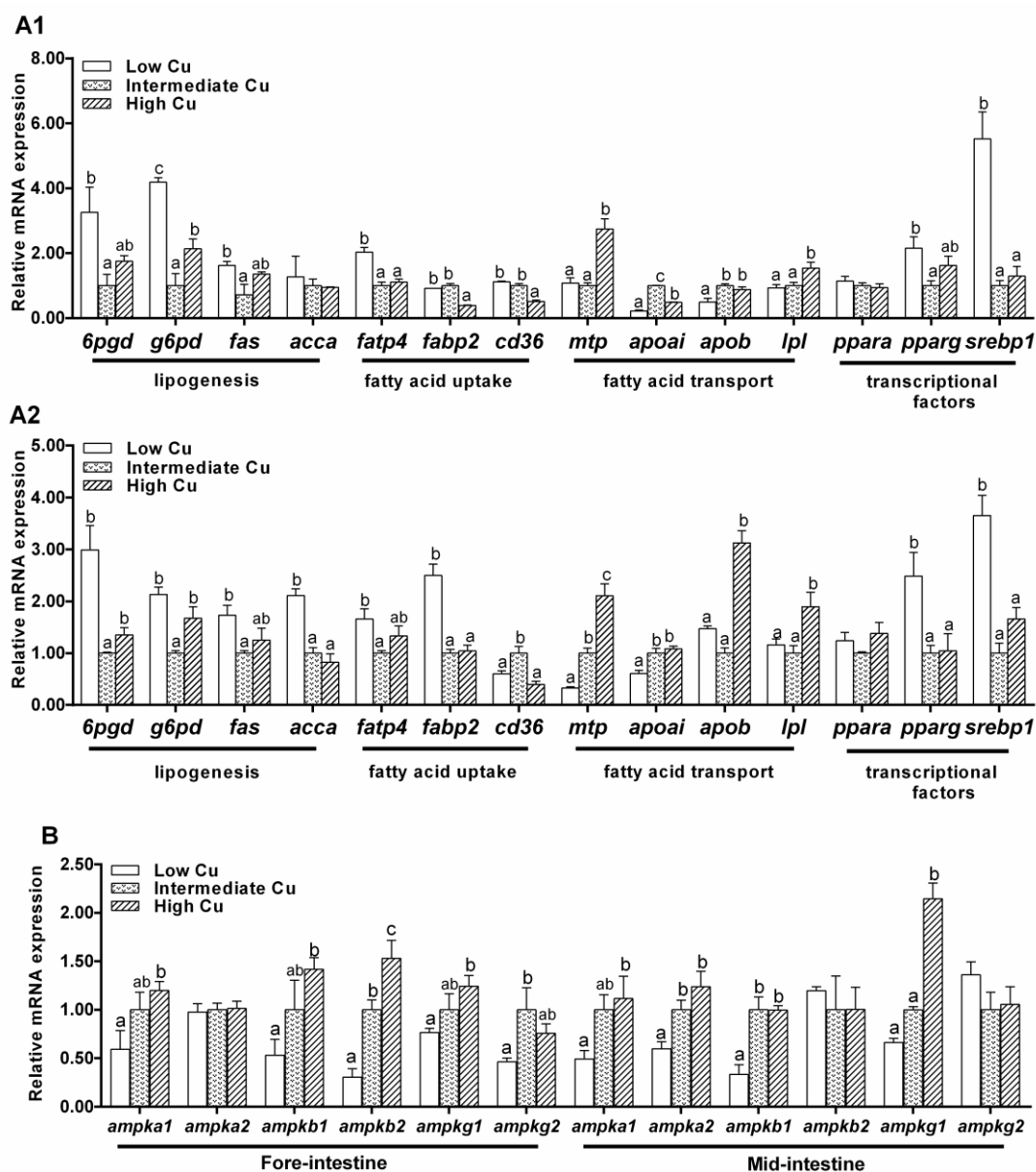


Fig. 2. Effect of different dietary Cu levels on the mRNA levels of genes involved in lipid metabolism (A) and AMPK signaling pathway (B) in the fore- and mid-intestine of yellow catfish. Values are mean \pm SEM (n= three replicate tanks, three fish were sampled for each tank). mRNA expression values were normalized to β -actin and *elfa* expressed as a ratio of the control (control=1). Different letters indicate significant differences among three treatments.

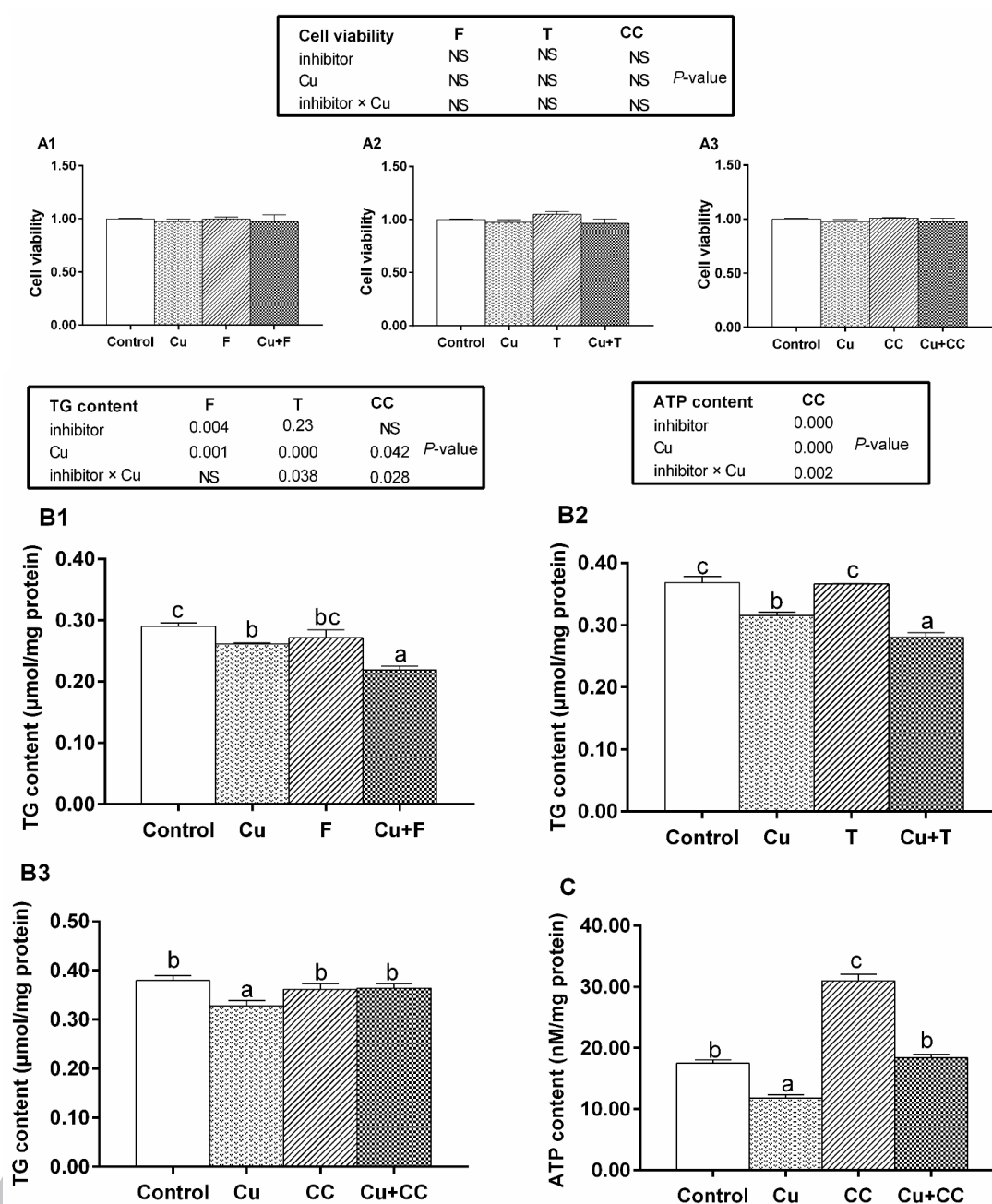


Fig. 3. Effects of Cu, Fatostatin, T0070907 and Compound C on cell viability (A1-A3) TG content (B1-B3) and ATP content (C) in primary enterocytes from yellow catfish. Data are presented as mean \pm SEM (n=3 independent biological experiments). Different letters indicate significant differences among the treatments.

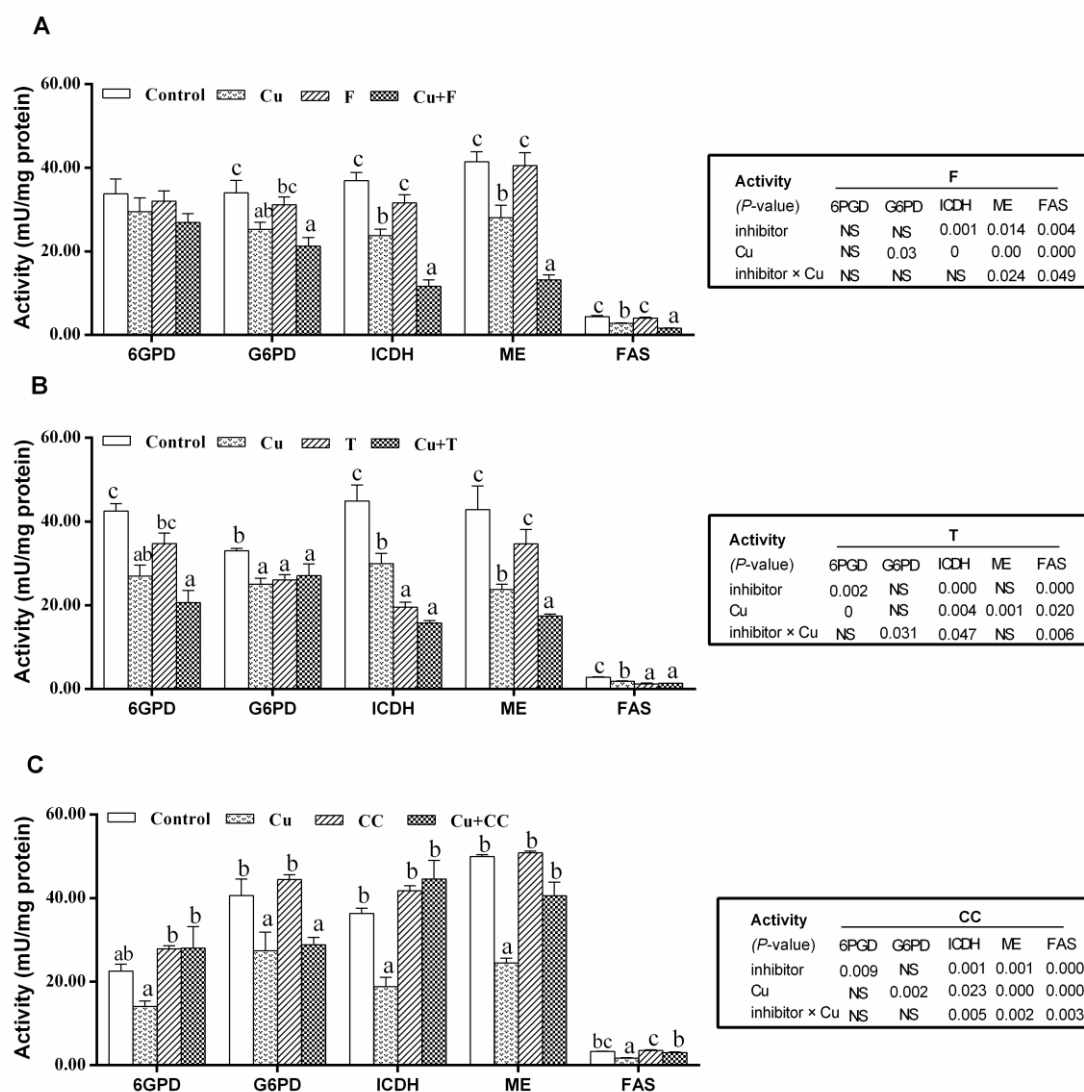


Fig. 4. Effects of Cu, Fatostatin (A), T0070907 (B) and Compound C (C) on enzyme activities involved in lipid metabolism in primary enterocytes of yellow catfish. Different letters indicate significant differences among the treatments.

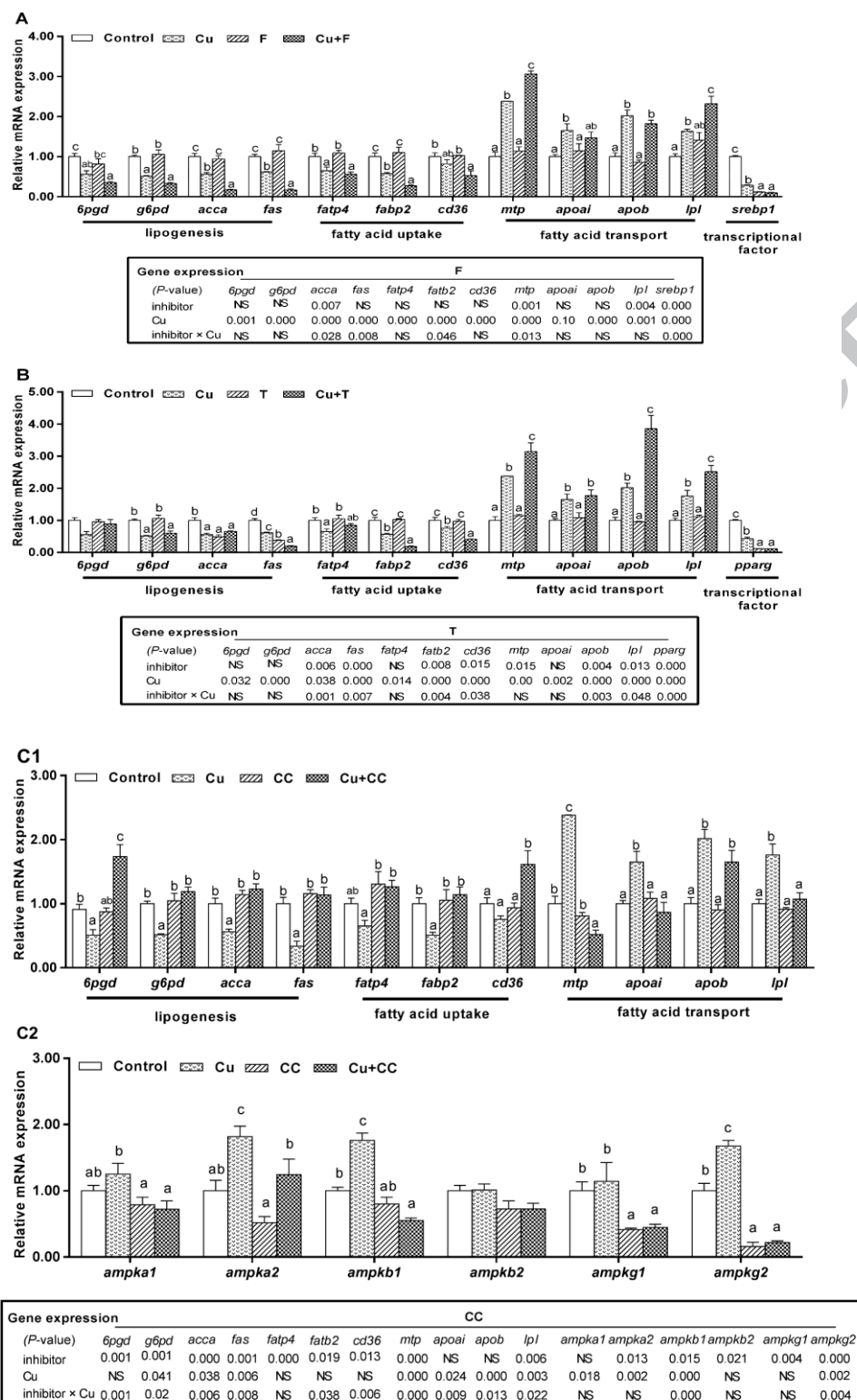


Fig. 5. Effects of Cu, Fatostatin (A), T0070907 (B) and Compound C (C) on gene expression involved in lipid metabolism (A, B, C1) and AMPK signaling pathway (C2) in primary enterocytes of yellow catfish. mRNA expression values were normalized to *18s* and *gapdh* expressed as a ratio of the control (control=1). Different letters indicate significant differences among the treatments.

Highlights

1. Dietary Cu triggered Cu accumulation but suppressed lipid deposition in the fore- and mid-intestine.
2. The reduced lipid deposition was attributable to the suppressed lipogenesis and lipid absorption, and accelerated lipid transport.
3. PPARG, SREBP1 and AMPK pathways mediated the Cu-induced changes in lipogenesis, lipid uptake and lipid transport.
4. Generally similar trend was observed in Cu-induced changes of lipid metabolism between the fore- and mid-intestine.